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Microbial Ecology of Retail Ready-to-Eat Escarole and Red Chicory Sold in Palermo City, Italy

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HIGHLIGHTS

- Escarole showed lower levels for all microbial groups than red chicory with the exception of the total yeast.
- The most numerous identified genera were *Pseudomonas* and *Pantoea*.
- Implementing good hygiene practices is needed during processing in order to prolong quality and acceptability of salads.

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Acronyms and abbreviations
RTE=Ready-To-Eat
CFU=Colony Forming Unit
TMM=Total Mesophilic Microorganisms
TPM=Total Psychrotrophic Microorganism
PCA=Plate Count Agar
CPS=Coagulase Positive Staphylococci
PCR=Polymerase Chain Reaction
RAPD=Random Amplification of Polymorphic DNA

ABSTRACT

Background: Ready-To-Eat (RTE) foods include any edible food that is commonly consumed raw. This study aimed at evaluation of microbial ecology of retail RTE escarole and red chicory sold in Palermo city, Italy.

Methods: A total of 32 mono-varietal RTE samples, including escarole (n=16) and red chicory (n=16) samples were obtained from Palermo, Italy. Both RTE vegetables at expiry date were analyzed to quantify spoilage bacteria, pathogenic bacteria, and yeast. All different colonies were isolated and identified on the basis of phenotypic characteristics and genetic polymorphisms by random amplification of polymorphic DNA-Polymerase Chain Reaction (PCR) and further genotype by sequencing the 16S rRNA gene. The statistical analysis was conducted with SAS 9.2 software (Statistical Analysis System Institute Inc., Cary, NC, USA).

Results: The level of *Listeria monocytogenes* and coagulase-positive staphylococci were below the detection. Total microbial counts were above 8 $\log_{10}$ colony forming unit/g in RTE red chicory, while they were about 1 log cycle lower in escarole. In general, escarole showed lower levels for all microbial groups than red chicory with the exception of the total yeast. A total of 13 strains were identified into ten species belonging to six genera as *Bacillus*, *Erwinia*, *Pantoea*, *Pseudomonas*, *Microbacterium*, and *Rahnella*. The most numerous identified genera were *Pseudomonas* and *Pantoea*.

Conclusion: This work pointed out the relevance of implementing good hygiene practices during processing in order to prolong quality parameters and acceptability of mono-varietal salads.

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Introduction

New trends in life style have led to a considerable increase of production, sale, and consumption of Ready-To-Eat (RTE) vegetables and fruits. This phenomenon is still on the increase because the consumers require more and more foods with high convenience of use and characterized by low-calorie. RTE fruits and vegetables provide high amounts of vitamins, minerals, fibres, and antioxidants such as polyphenols, flavonoids, sterols, carote

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noids, chlorophyll, anthocyanins, etc. (Giugliano and Esposito, 2008; Siriamornpun et al., 2012). Furthermore, their consumption is encouraged in many countries of the globe by national government health agencies in order to prevent a range of diseases such as cancer and cardiovascular illnesses (Mercanoglu Taban and Halkman, 2011).

RTE vegetables are processed from the fresh products basically through selection, prewashing, cutting, washing, drying, and packaging (de Oliveira et al., 2011). This processing, in most cases, is insufficient to ensure the microbiological stability of the final products. The main factors affect on the occurrence of microbial contaminations in vegetables include water, soil, manure of wild, and domestic animals (Miceli and Settanni, 2019) and the erroneous application of Good Manufacturing Practice (GMP) and Hazard Analysis and Critical Control Point (HACCP) systems during any step of production, distribution, and administration to consumers (De Giusti et al., 2010). RTE vegetables are more susceptible to microbial contamination because the natural protective barrier provided by epidermidis is removed during the cutting operations, favor the release of nutrients and facilitate the growth of microorganisms (Abadias et al., 2008; Puerta-Gomez et al., 2013). Furthermore, rapid growth of pathogenic microorganisms in these products represents a severe health issue to consumers. The implicate microorganisms include *Pseudomonas* spp., *Xanthomonas* spp., *Enterobacter* spp., *Chromobacterium* spp., lactic acid bacteria, yeasts, and occasionally *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. (Lavelli et al., 2009; Mercanoglu Taban and Halkman, 2011). Furthermore, the microbiological investigations of RTE vegetables and sprouts in several countries have revealed that these matrices have been the cause of death in USA and northern Europe (Abadias et al., 2008). Among leafy vegetable, escarole (*Cichorium endivia* var. *latifolium*) and red chicory (*Cichorium intybus* L.) are available in pre-packed fresh cut form with a storage life of about 7–10 days under refrigeration condition (Tsironi et al., 2017).

So far, few studies have been conducted to evaluate the microbiological quality of RTE mono-varietal vegetables such as spinach, broccoli, lettuce, escarole, red chicory, etc. (Alfonzo et al., 2018; Bencardino et al., 2018; Miceli et al., 2019). In the present work, the microbial ecology of packed mono-varietal RTE escarole and red chicory was investigated at expiry date using samples cultivated, transformed into RTE, and commercialized in a restricted area, represented by Palermo city (Sicily, Italy), in order to exclude the effect of transport, since it represented a "zero kilometre" vegetable food production.

Materials and methods

Sample collection

A total of 32 mono-varietal RTE samples, including escarole (n=16) and red chicory (n=16) samples were obtained from Palermo, Italy. Soon after purchasing, all samples were placed into a portable fridge and transferred to the Laboratory of Agricultural Microbiology (Department of Agricultural, Food and Forestry Science, University of Palermo) and kept at 4 °C until the expiry date indicated on the labels (9 days from production). RTE vegetable collection was carried out in duplicate and repeated after two weeks.

Microbiological analyses

Twenty-five g of cut leaves from each RTE vegetable were transferred into sterile plastic bags (BagLight^{R} 400 Multilayer^{R} bags, Interscience, Saint Nom, France), added with Ringer's solution (225 ml) (Sigma Aldrich, Milan, Italy), and homogenised in a stomacher (Bag-Mixer 400; Interscience) for 2 min at the maximum speed (blending power 4). All homogenized samples were then subjected to the decimal serial dilution procedure. Cell suspensions were plated and incubated as follows: Total Mesophilic Microorganisms (TMM) on Plate Count Agar (PCA), incubated at 30 °C for 72 h; Total Psychrotrophic Microorganisms (TPM) on PCA , incubated at 7 °C for 7 days; pseudomonads on *Pseudomonas* Agar Base (PAB) added with cetrimide fucidin cephaloridine supplement, incubated at 25 °C for 48 h; members of the Enterobacteriaceae family on Violet Red Bile Glucose Agar (VRBGA), incubated at 37 °C for 24 h; total coliforms on Violet Red Bile Agar (VRBA), incubated at 37 °C for 24 h; enterococci on Kanamycin Aesculin Azide (KAA) agar, incubated at 37 °C for 24 h; Coagulase-Positive- and Coagulase-Negative Staphylococci (CPS and CNS) on Baird Parker (BP) added with rabbit plasma fibrinogen supplement, incubated aerobically at 37 °C for 48 h; *L. monocytogenes* on *Listeria* Selective Agar Base (LSAB) added with SR0140E supplement, incubated at 37 °C for 48 h; yeasts on Yeast extract Peptone Dextrose (YPD) agar supplemented with 0.1 g/l chloramphenicol to avoid bacterial growth, incubated at 30 °C for 48 h. All media and supplements were purchased from Oxoid (Milan, Italy). All microbiological counts were done in triplicate.

Isolation and grouping of bacteria

After growth, at least five colonies with the same appearance (colour, morphology, edge, surface, and elevation) were randomly picked up from petri dishes and transferred into the corresponding broth media. The

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isolates were purified by successive subculturing on the same media used for plate counting and their purity was verified by optical microscope (Olympus, BX60, Japan).

In order to perform a phenotypic grouping of bacteria, all isolates were preliminary characterized. The KOH test (Gregersen, 1978) was performed to determine the type of cell wall. The catalase test was carried out by direct addition of H_2O_2 (5%, w/v) to the colonies. Cell morphology and motility were evaluated by microscopic inspection. The formation of spores was investigated as follows: the cell suspensions of the pure cultures were treated at 85 °C for 15 min and, subsequently, inoculated in the same media used for isolation and purification, then incubated at the optimal growth conditions.

Genotypic differentiation and identification of bacteria

DNA extraction from cells was performed by lysis using the Instagene Matrix kit (Bio-Rad, Hercules, CA) following the manufacturer's instruction. Crude cell extracts were used as templates for Polymerase Chain Reaction (PCR).

Strain differentiation was performed with Random Amplification of Polymorphic DNA (RAPD)-PCR analysis in a 25 µl reaction mixture using the single primers M13, AB111, and AB106 as previously described by Gaglio et al. (2017). Each reaction mixture contained 0.2 mM of dNTP mix (Life Technologies Italia, Monza, Italy), 0.625 U of Taq DNA polymerase (Fermentas, MMedical, Milan, Italy), 2.5 µl of PCR buffer (Fermentas, Italy), 2.5 mM of $MgCl_2$, 2 µM of each primer, 2.5 ng of DNA, and Milli-Q® water (Millipore, Billerica, MA, USA) to reach the final reaction volume. The amplifications were performed using a T1 Thermocycler (Biometra, Göttingen, Germany) to generate amplicons which were visualized by UV transillumination and acquired by Kodak EDAS 290 (Rochester, USA). The software package Gelcompare II Version 6.5 (Applied Maths, Sin-Martens-Latem, Belgium) was used to analyze the band patterns.

All bacteria showing different RAPD-PCR profiles were considered different strains and analyzed by 16S rRNA gene sequencing for identification at species level. PCRs were performed as described by Weisburg et al. (1991) using the primers rD1 (5'-AAGGAGGTGATCC AGCC-3') and fD1 (5'-AGAGTTTGATCCTGGCTCAG -3') in a 50 µl reaction volume. PCR mixture contained 0.25 mM of dNTP mix (Life Technologies Italia), 1.5 U of Taq DNA polymerase, 3 µl of PCR buffer (Fermentas, Italy), 3 mM of $MgCl_2$, 0.2 µM of both primers, 25 ng of DNA, and was brought to the final volume with Milli-Q® water (Millipore). DNA fragments were run and visualized as reported above. The amplicons corresponding approximately to 1400 bp were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). DNA sequencing reactions were performed with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Beverly, MA) with 5 µM of each primer used for DNA amplification. Cycle sequencing reactions were performed according to the manufacturer's instructions following ethanol-EDTA-sodium acetate precipitation. Sequencing analyses were done in an ABI Prism 3130xl genetic analyzer (Applied Biosystems, USA).

The sequences obtained for each strain were compared with those available in EzTaxon-e database (http://eztaxon-e.ezbiocloud.net/) that includes only 16S rRNA gene sequences of the strain types and with those deposited in GenBank/EMBL/DDBJ (http://www.ncbi. nlm.nih.gov; https://www.ddbj.nig.ac.jp/index-e.html; https://www.embl.de).

Statistical analyses

Microbiological data were subjected to one-way Analysis of Variance (ANOVA). Pair comparison of treatment means was achieved by Duncan procedure at $p<0.05$. Differences between RTE escarole and RTE red chicory were evaluated with the Generalised Linear Model (GLM) procedure. The statistical analysis was conducted with SAS 9.2 software (Statistical Analysis System Institute Inc., Cary, NC, USA).

Results

Microbial loads of packed mono-varietal RTE escarole and red chicory samples at the expiry date are reported in Figure 1. Statistical significant differences were found for the levels of TPM (p=0.021), TMM (p=0.018), pseudomonads (p=0.045), and total coliforms (p=0.004) between the two salads analyzed.

None of the samples object of investigation was scored positive for the presence of enterococci, CPS, and *Listeria* spp. For both RTE vegetables, the levels of TPM, TMM, and pseudomonas were comparable and represented the microbial groups seen at the highest cell densities after the refrigerated storage. In particular, the levels of these microbial groups were above 8 $\log_{10}$ Colony Forming Unit (CFU)/g in RTE red chicory, while they were about 1 log cycle lower in escarole. Generally, escarole showed lower levels for all microbial groups than red chicory with the exception of the total yeast.

A total of 252 bacterial isolates were separated into three groups according to colony, microscopic morphology, and preliminary biochemical tests (Table 1). Only straight rod cell morphologies were observed. Only bacteria included in group 3 constituted the most numerous group with more than 77% of total isolates which were

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Gram-negative. Bacteria included in groups 1 and 2 were non-motile. The group 1 bacteria were spore forming.

All bacterial cultures were subjected to RAPD-PCR analysis for strain typing. As reported in the dendrograms (Figure 2), the combination of three RAPD patterns of each isolate indicated that the cultivable bacterial community was composed of 13 different strains which associated to the RTE escarole and red chicory analyzed in this study at the expiry date.

These strains were identified by sequencing of the 16S rRNA gene and the sequences were compared with those available in two distinct databases by BLAST and EZ-taxon search; all strains were clearly identified with a similarity higher than 99% in both databases (Table 2) and they were allotted to six genera. The most numerous genera were *Pseudomonas* and *Pantoea*, including four strains representing three species, and *Bacillus*, including two strains representing one species, followed by *Erwinia*, *Microbacterium*, and *Rahnella* with one strain per species (Figure 2). All 13 sequences of 16S rRNA gene were deposited in GenBank as follows: *B. clausii*, Ac. No. MK496619–MK496620; *E. rhapontici*, Ac. No. MK496621; *M. binotii*, Ac. No. MK496622; *P. ananatis*, Ac. No. MK496623; *P. anthophila*, Ac. No. MK496624; *P. eucalypti*, Ac. No. MK496625–MK496626; *Ps. azotoformans*, Ac. No. MK496627–MK496628; *Ps. psychrophila*, Ac. No. MK496629; *Ps. poae*, Ac. No. MK496630; *R. victoriana*, Ac. No. MK496631. Table 2 shows the highest biodiversity in the samples of escarole that hosting seven different species. Regarding the distribution of the genera, *Bacillus*, *Microbacterium*, and *Erwinia* species were found just in escarole samples, *Rahnella* was found in red chicory samples, and *Pantoea* and *Pseudomonas* were found in both samples.

The dendrogram illustrated the strains grouped per genus and species. A major RAPD-PCR cluster was observed for the phenotypic group 3 which included all Gram-negative and catalase positive bacteria (*Erwinia*, *Pantoea*, *Pseudomonas*, and *Rahnella*).

Table 1: Phenotypic grouping of bacteria isolated from mono-varietal RTE escarole and red chicory

Characters	Groups		
	1 (n=47)	2 (n=13)	3 (n=192)
Cell morphology	Straight rod	Straight rod	Straight rod
Gram reaction	Positive	Positive	Negative
Catalase test	Positive	Positive	Positive
Motility	–	–	+
Spore formation	+	–	–

Table 2: Identification of bacteria strains isolated from mono-varietal RTE escarole and red chicory

Strain	Genus and species	Sample	Phenotypic group	% Similarity (accession no. of closest relative) by:		Sequence length (bp)
				BLAST	EzTaxon	
4G225	*Bacillus clausii*	Escarole	1	99 (KR632489.1)	99.15 (DSM 8716)	1443
4G219	*Bacillus clausii*	Escarole	1	99 (MK312486.1)	99.31 (DSM 8716)	1456
4G186	*Erwinia rhapontici*	Escarole	3	99 (KR054974.1)	99.51 (ATCC 29283)	1430
4G222	*Microbacterium binotii*	Escarole	2	99 (JQ291602.1)	99.93 (CIP 101303)	1422
4G241	*Pantoea ananatis*	Escarole	3	99 (DQ133546.1)	99.09 (LMG 2665)	1430
4G239	*Pantoea anthophila*	Red chicory	3	99 (JQ659513.1)	99.57 (LMG 2558)	1427
4G233	*Pantoea eucalypti*	Escarole	3	99 (HM008959.1)	99.63 (LMG 24198)	1429
4G247	*Pantoea eucalypti*	Red chicory	3	99 (JQ446440.1)	99.55 (LMG 24198)	1412
4G182	*Pseudomonas azotoformans*	Escarole	3	99 (MK026816.1)	99.65 (DSM 18862)	1423
4G263	*Pseudomonas azotoformans*	Escarole	3	100 (MK026816.1)	99.72 (DSM 18862)	1424
4G202	*Pseudomonas psychrophila*	Red chicory	3	99 (KY939756.1)	99.65 (E-3)	1424
4G250	*Pseudomonas poae*	Escarole	3	100 (KY810658.1)	99.72 (DSM 14936)	1424
4G195	*Rahnella victoriana*	Red chicory	3	100 (KY939761.1)	99.78 FRB 225	1431

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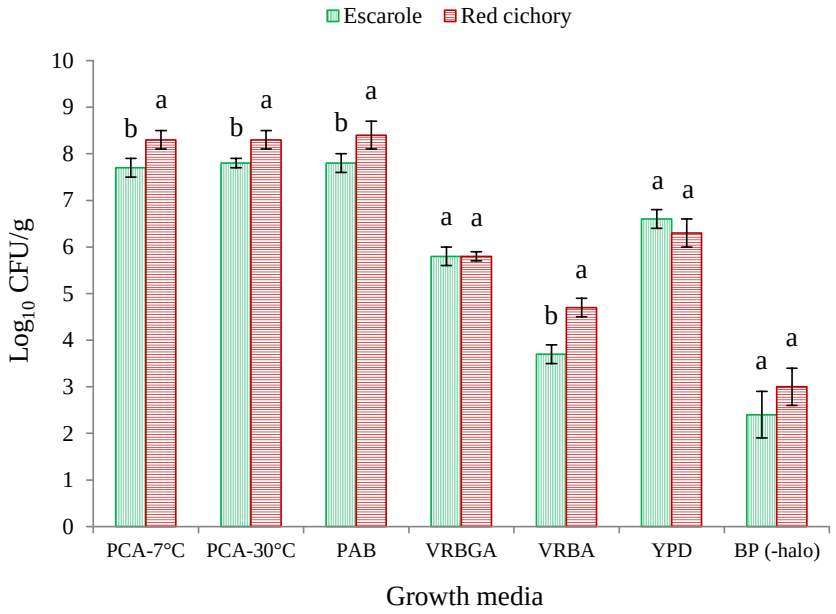


Figure 1: Microbial loads (log_{10} CFU/g) of mono-varietal RTE escarole and red chicory. PCA-7 °C, Plate Count Agar for detection of total psychrotrophic microorganisms; PCA-30 °C, Plate Count Agar for detection of total mesophilic microorganisms; PAB, *Pseudomonas* Agar Base for detection of pseudomonads; VRBGA, Violet Red Bile Glucose Agar for detection of Enterobacteriaceae; VRBA, Violet Red Bile Agar for detection of total coliforms; YPD, Yeast extract Peptone Dextrose agar for detection of yeast; BP, Baird Parker for detection of coagulase-positive and coagulase-negative staphylococci. Different superscript letters indicate significant differences on microbial concentrations were performed at each sampling according to Duncan test between RTE escarole and red chicory samples for $p<0.05$

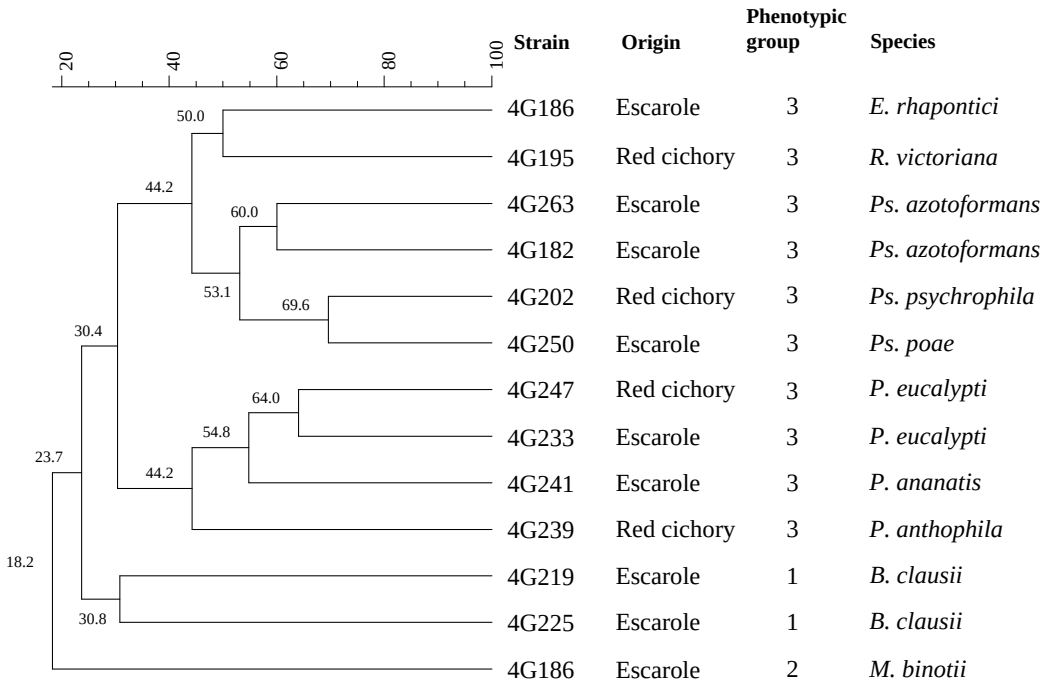


Figure 2: Dendrogram obtained with combined RAPD-PCR patterns in this study. *B.*: *Bacillus*; *E.*: *Erwinia; M.*: *Microbacterium*; *P.*: *Pantoea*; *Ps.*: *Pseudomonas*; *R.*: *Rahnella*

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Discussion

According to the definition given by Food and Agriculture Organization and World Health Organization, RTE foods include any edible food that is commonly consumed raw (FAO and WHO, 2004). As RTE vegetables do not need further preparation before consumption, the presence of pathogens as part of their indigenous microflora might pose public health concerns (Cerna-Cortes et al., 2015). Recently, due to the increased consumption of RTE vegetables, they have been object of several researches focusing on their hygienic properties (Zare Jeddi et al., 2014). This is the first work focused on the microbial ecology of packed mono-varietal RTE escarole and red chicory produced and commercialized in the same city. All vegetable samples analyzed in this study were cultivated, transformed into RTE and sold in Palermo (Italy). The choice of considering a "zero kilometre" production depended on the fact that transport can influence microbiological characteristics of fruits and vegetables, even if the cold chain is strictly applied (La Scalia et al., 2016, 2017).

For both escarole and red chicory samples in this survey, the evolution of the microbiological parameters was estimated at the expiry date (9 days after production) indicated on the labels. The microbiological analyses evidenced statistical differences among four of ten microbial groups investigated for two mono-varietal salads. The levels of TPM, TMM, and pseudomonads were found at the highest cell densities in the vegetables as reported in Tehran, Iran (Zare Jeddi et al., 2014), Palermo, Italy (Alfonzo et al., 2018), Avezzano, Italy (Lavelli et al., 2009), Palermo, Italy (Miceli et al., 2019), and Athens, Greece (Tsironi et al., 2017). All these study reported levels of aerobic microbial groups always lower than 7 $\log_{10}$ CFU/g. Similarly, analysis of RTE commercial vegetables in Brazil carried out by de Oliveira et al. (2011) showed that TPM count was >5 log CFU/g. Generally, these aerobic microbial groups are not related to food poisoning and infections. However, a recent investigation conducted in Palermo by Francesca et al. (2018) showed that *Pseudomonas* are involved in antibiotic resistance to ampicillin and amoxicillin commonly used to the treatment of human and animal infections. We found that yeasts were 1 and 2 log cycles lower than pseudomonads for escarole and red chicory, respectively. In general, pseudomonads and yeasts are commonly associated to the spoilage of RTE salads (Tsironi et al., 2017). Enterococci and CPS did not generate colony in any sample. Furthermore, in light of the Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs (Commission Regulation, 2005), *L. monocytogenes* was never detected in any sample. However, the presence of these pathogens microorganisms may represent a serious threat to the consumers (Bencardino et al., 2018). For instance, *Listeria* spp. was detected in 3.7% of Brazilian RTE vegetables (de Oliveira et al., 2011) which may endanger local public health.

None of the *Pseudomonas* species identified in this study is reported to be pathogenic for humans. All species identified are associated to soil, plant roots, sludge or water. Hence, their presence indicates an environmental contamination (Franzetti and Scarpellini, 2007). In the previous researches, *Ps. azotoformans* was isolated from whole vegetables of red chicory (Alfonzo et al., 2018), RTE escarole processed without the cutting operation (Miceli and Settanni, 2019) and food ice (Settanni et al., 2017). Also, *Ps. psychrophila* was detected from sewage effluent (Jiang et al., 2012), cold storage (Yumoto et al., 2001) and Arctic circle (Abraham and Thomas, 2015); and *Ps. poae* was found in phyllosphere of grasses (Behrendt et al., 2003).

All *Pantoea* species identified in the current investigation are usually isolated from soil, fruit, vegetables (De Baere et al., 2004) and represent a possible cause of plant pathology (Brady et al., 2009). Other bacterial species identified from packed mono-varietal RTE escarole and red chicory may be originated from food and environment. *B. cereus* is a spore-forming pathogenic bacteria commonly associated to dairy products, jennet milk, pasteurized egg yolk, cooked rice, and vegetable (Choma et al., 2000; Scatassa et al., 2011). *M. binotii*, recently described from cellulolytic gut flora of beetle larvae (Huang et al., 2012) causing bacteremia in a patient with sickle cell anemia (Buss et al., 2014). Also, *R. victoriana* was isolated from oak tissue affected by the acute oak decline and involved in tissue decay of plants (Brady et al., 2014).

Conclusion

Packed mono-varietal RTE escarole and red chicory analyzed in this study were characterized by quite satisfactory microbiological quality compared to the previous reports. Several identified species were commonly associated to the spoilage of RTE salads, but were never pathogenic for human. However, this study highlighted the need to implement good hygiene practices during processing in order to prolong quality parameters and acceptability. To this purpose, applied transformation processes as well as transport conditions could be object of investigation in order to keep under control additional contamination sources.

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Author contributions

R.Ga. and L.S. designed the project of study; N.F., R.Gu., V.C., and R.Ga. conducted the experiments; R.Ga. and G.M. analyzed the data; R.G. and L.S. wrote the manuscript. All authors revised and approved the final manuscript.

Conflicts of interest

There was no conflict of interest in this work.

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